Mitochondrial-Disrupting ToxCast Chemicals Inhibiting the Electron Transport Chain

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2439-P854

Introduction

- Mitochondria generate >90% of eukaryotic cellular ATP via oxidative phosphorylation (OXPHOS) (Fig. 1)
  - Also important in regulation of cell proliferation, metabolic regulation, and apoptosis
- Mitochondrial dysfunction is implicated in several adverse outcomes:
  - Parkinson's and Alzheimer's Disease
  - Metabolic syndrome
  - Cancer
  - Diabetes
- Chemicals in the environment are known to impair mitochondrial function through various mechanisms:
  - Electron transport chain inhibition (ETC; complexes I – IV)
  - Uncoupling of the inner mitochondrial membrane (IMM)
  - Inhibition of phosphorylation through ATP synthase
  - ATP transport inhibition
  - Krebs cycle inhibition
- Previous ToxCast mitochondrial screening assays focused on mitochondrial mass or membrane potential
  - Dye-based assays may be less sensitive to chemicals that functionally impair mitochondria
- As US EPA seeks to develop high-throughput screening methods to broaden the chemical knowledge space and improve model accuracy, two new ToxCast assays measure respiration (oxygen consumption rate; OCR) using the Agilent Seahorse XFe96 Analyzer to identify putative mitochondrial-disrupting chemicals and confirm mechanisms of action

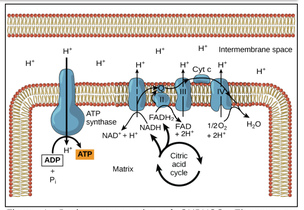

Figure 1: Basic representation of OXPHOS. Electrons are transferred along 4 ETC complexes while H⁺ ions are pumped across the IMM generating a proton gradient to drive ATP synthase. Disruption of this process can lead to adverse effects. Image: OpenStax CNX.

Methods: Intact-Cell Mitochondrial Respirometric Assay

- Hepatocellular carcinoma cell line HepG2 generates ~50% ATP via OXPHOS (data not shown)
  - Many immortalized cell lines rely heavily upon glycolysis for ATP production
- Cellular OCR indicative of mitochondrial function measured with Agilent Seahorse XFe96 Analyzer
- Four temporal windows separated by three sequential reagent additions (Fig. 2):
  1. Pre-injection respiration (OCR)
  2. Injection 1: Basal respiration – control (vehicle, ETC inhibitor, IMM uncoupler) or test chemical exposure
  3. Injection 2: Maximal respiration induced with known IMM uncoupler (FCCP) exposure
  4. Injection 3: Inhibited respiration induced with known ETC inhibitor cocktail (rotenone/antimycin A) exposure
- Test chemical exposure is 18 minutes before maximal respiration is induced with IMM uncoupler
- ToxCast Phase I and II chemicals (1,051 blinded test samples) were initially assayed at 100µM only
- Activity thresholds were set using OCR variability of vehicle wells for each temporal window (Fig. 2)
- Chemicals active at 100µM were retested at 7 concentrations ranging 0.125 - 100µM
- Chemicals were then categorized based on putative mechanism (Fig. 3B)
- Twenty potential ETC inhibitors were subsequently assessed using an electron flow assay

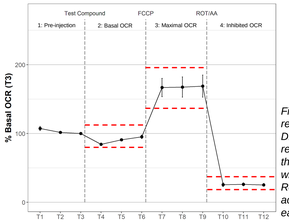

Figure 2: Graph representing normalized DMSO response to respirometric assay over the four temporal windows of exposure. Red-dotted lines indicate activity thresholds for each window.

Methods: Permeabilized-Cell Mitochondrial Electron Flow Assay

- Permeabilize HepG2 cells with Agilent plasma membrane permeabilizer (PMP) for direct exposure to mitochondria
- Initiate maximal respiration through ETC complex I by supplying pyruvate and malate with IMM uncoupler (FCCP) exposure
- Mitochondrial OCR measured with Agilent Seahorse XFe96 Analyzer
- Four temporal windows separated by three sequential reagent additions (Fig. 5):
  1. Baseline maximal respiration (OCR)
  2. Injection 1: Controls (vehicle, ETC inhibitors) or test chemicals (at 31, 64, and 100µm) for 18 minutes
  3. Injection 2: Simultaneously block complex I (rotenone) and restore respiration through complex II with succinate
  4. Injection 3: Simultaneously block complex III (antimycin A) and restore respiration at complex IV with ascorbate/TMPD
- Qualitatively assign putative ETC complex inhibition mechanism to test chemical based on OCR recovery/non-recovery

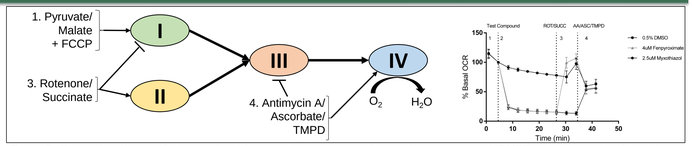

Figure 5: Schematic of sequential reagent additions and graph representing control responses of DMSO vehicle, complex I inhibitor fenpyroximate, and complex III inhibitor myxothiazol within the four temporal windows of exposure.

Results: Permeabilized-Cell Mitochondrial Electron Flow Assay

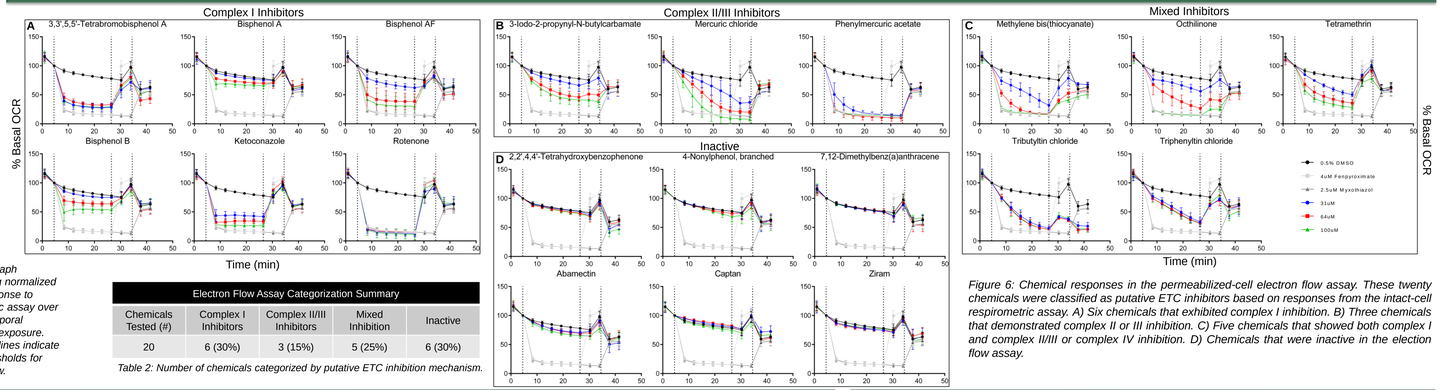

Figure 6: Chemical responses in the permeabilized-cell electron flow assay. These twenty chemicals were classified as putative ETC inhibitors based on responses from the intact-cell respirometric assay. A) Six chemicals that exhibited complex I inhibition. B) Three chemicals that demonstrated complex II or III inhibition. C) Five chemicals that showed both complex I and complex II/III or complex IV inhibition. D) Chemicals that were inactive in the election flow assay.

Electron Flow Assay Categorization Summary				
Chemicals Tested (#)	Complex I Inhibitors	Complex II/III Inhibitors	Mixed Inhibition	Inactive
20	6 (30%)	3 (15%)	5 (25%)	6 (30%)

Table 2: Number of chemicals categorized by putative ETC inhibition mechanism.

Results: Intact-Cell Mitochondrial Respirometric Assay

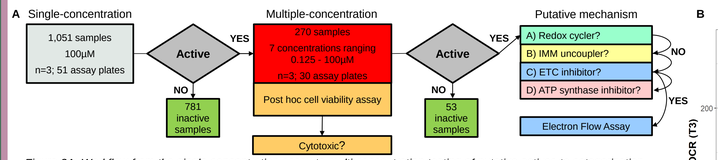

Figure 3A: Workflow from the single-concentration assay to multi-concentration testing of putative actives to categorization of potential mechanisms of action. Of 1,051 tested samples, 834 were ultimately deemed inactive while 217 were further characterized (Figure 3B). A majority of actives were classified as potential ETC inhibitors. Twenty of these ETC inhibitor candidates were assessed using an electron flow assay.

Multi-concentration Respirometric Assay Categorization Summary					
Chemicals Tested (#)	Redox-cyclers	IMM uncouplers	ETC inhibitors	ATP synthase inhibitors	Inactive
270	10 (4%)	16 (6%)	161 (60%)	30 (11%)	53 (20%)

Table 1: Number of chemicals categorized by putative mechanism of mitochondrial disruption.

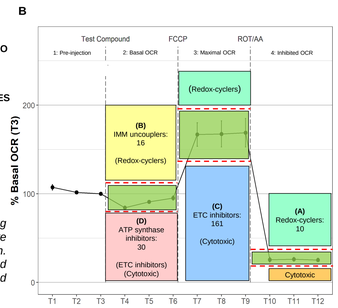

Figure 3B: Graphical representation showing how active responses in windows of exposure were used for mechanistic categorization. Redox-cyclers (A) were identified first, followed by IMM uncouplers (B), ETC inhibitors (C), and then ATP synthase inhibitors (D).

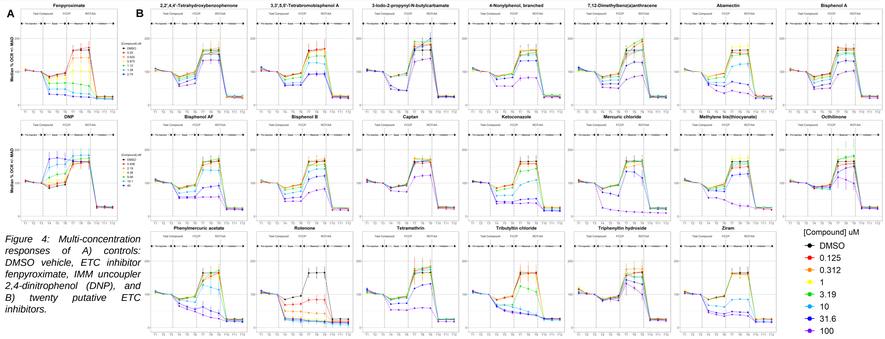

Figure 4: Multi-concentration responses of A) controls: DMSO vehicle, ETC inhibitor fenpyroximate, IMM uncoupler 2,4-dinitrophenol (DNP), and B) twenty putative ETC inhibitors.

Conclusions/Future Directions

- Mitochondrial oxygen consumption rates (OCR) of intact HepG2 cells were measured over four temporal windows: pre-injection, basal, maximal and inhibited OCR.
  - Using DMSO variability as a threshold of active response within each window allowed for categorization of chemical putative mechanism of action.
  - Of 1,051 samples, 834 ultimately tested inactive while among the 217 active samples, most (161) were putative electron transport chain (ETC) inhibitors.
  - Twenty potential ETC inhibitors were further characterized in an electron flow assay to determine if a specific ETC complex (I – IV) was affected.
- Maximal mitochondrial OCR of permeabilized HepG2 cells was then measured over four temporal windows: baseline maximal respiration, chemical exposure, complex I inhibition/complex II recovery, and complex III inhibition/complex IV recovery.
  - Using OCR recovery/nonrecovery as a basis for categorization, six chemicals specifically blocked complex I, three blocked complex II/III, five inhibited multiple complexes, and the remaining six were inactive.
- This is the first large-scale effort to identify and confirm mechanisms of chemically-induced mitochondrial dysfunction using a screening-based approach.
- All suspected ETC inhibitors within the ToxCast Phase I and II chemical libraries will be subsequently tested using the electron flow assay to confirm their activity and identify their target ETC complex(es).
- These data will expand the knowledge base for mitochondrial toxicants and may ultimately assist the Agency in decision-making processes.